REMARKS

The Specification has been amended to contain a specific reference to the priority document as required under 35 U.S.C. 119(e) and an abstract of the disclosure under 37 C.F.R. 1.72(b).

Claims 11, 12 , 13 and 19 have been cancelled. Claim 18 has been amended to correct an obvious typographical error. Original Claims 11 and 12 have been reinstated as Claims 26 and 28. New Claims 27 and 29 have been added to reinstate the subject matter claimed in original Claim 13 (now cancelled). Claims 1-10 and 14-18 and 20-29 are pending in the present application. Claims 1-5 and 10-24 stand rejected and claims 6-9 and 25 stand objected to.

No new matter has been added. Reconsideration and reexamination of the present application in view of the amendments and remarks presented herein is respectfully requested.

Rejection of Claims 1-5 and 10-24 Under 35 U.S.C. 112, Second Paragraph

Claims 1-5 and 10-24 are rejected under 35 U.S.C. 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention for reasons made of record. This rejection is respectfully traversed.

First, the Action alleges that the term, "4-substituted piperazine" is indefinite. Applicants respectfully direct the Examiner's attention to the specification, page 6, line 20, which states that piperazinyl is included in the definition of the term heterocycle. Furthermore, at lines 15-18, it is stated that a heterocycle (which by definition includes piperazine) may be substituted with up to three substituents (and in this case it would be one substituent in the 4-position) independently selected from the group consisting of halo, C_1-C_4 alkoxy, C_1-C_4 alkyl, cyano, nitro, hydroxy, -$S(O)_m$-(C_1-C_4 alkyl) and –$S(O)_m$-phenyl where m is 0, 1, or 2. Thus, a 4-substituted piperazine would be a piperazine substituted in the 4-position with one of these substituents. Therefore, the term, "4-substituted piperazine" is not indefinite and Applicants respectfully request the withdrawal of this rejection.

Second, Applicants have cancelled claims 11-13, thereby obviating the rejection of Claims 11 and 12 under points 2 and 3 of the Action.

Third, the Action alleges that it is not clear what actual process "neuronal protein extravasation" refers to and requests the submission of a scientific article on the subject. As

requested, Applicants submit her with copies of two such papers on the subject, each of which also reference many other papers:

Johnson, Kirk W., et al., "5-HT1F receptor agonists inhibit neurogenic dural inflammation in guinea pigs", Neuroreport, vol. 8, pgs. 2237-2240 (1997); and

Johnson, Kirk W., and Phebus, Lee A., "A fluorescence-based method for assessing dural protein extravasation induced by trigeminal ganglion stimulation", J. Neuroscience Methods, vol. 81, pgs. 19-24 (1998).

Finally, the Action indicates that the term "nitronium ion" is not present in claim 14 and asks what a "nitronium ion" is. According Streitwieser and Heathcock, *Introduction to Organic Chemistry*, pgs. 586-587, 1976, a text well known to those skilled in the art, a nitronium ion means NO_2^+. Thus, one skilled in the art reading claim 14 would clearly understand what the term "nitronium ion" means. A copy of the referenced pages is attached for the Examiner's convenience.

Regarding the appearance of the term, "nitronium ion" is inherently present in step (b) of Claim 14 where it states the process term "nitrating." That this inherency is commonly understood by one skilled in the art, can be appreciated from the discussion of nitration in the cited Streitwieser & Heathcock reference.

Furthermore, the Examiner is referred to the specification at page 31, line 29, through page 32, line 28, where it states that after treatment with the source of the protecting group [step a], the N-protected benzoylpiperidine is treated with the source of nitronium ion to form a mixture or N-protected 4-(mononitronbenzoyl)piperidines (step b). The referenced section then goes on to state that the source of the nitronium ion useful for the process of step (b) of the invention include fuming nitric acid and inorganic nitrate salts, preferably ammonium nitrate. Thus, it is clear to one skilled in the art reading claim 14 in view of the specification that the term "nitronium ion" is inherently present in the process term "nitrating". Applicants respectfully request the withdrawal of this rejection.

In view of these amendments and arguments, withdrawal of the present rejection is believed to be in order and such action is respectfully requested.

Rejection of Claims 1, 2, 4,(5,) and 10-12 Based on 35 U.S.C. §103(a)

Claims 1, 2, 4, 5, and 10-12 are rejected under 35 U.S.C. 103(a) as being unpatentable over Carr. Additionally, claims 1-5 and 10-12 are rejected as being unpatentable over Butera, Oinuma, or Helsley, alone or in view of Carr. This rejection is respectfully traversed.

Applicants submit herewith the declaration of Dr. John M. Schaus demonstrating the unexpected and surprisingly high $5\text{-}HT_{1F}$ receptor affinity arising from the substitution of the benzene ring of the compound of formula I at the 3-position rather than the 4-position. In light of this declaration, Applicants respectfully request the withdrawal of these rejections.

Missing Abstract

Applicants hereby submit an abstract of the disclosure as required by 37 CFR 1.72(b).

Objection to Claims 6-9 and 25

Claims 6-9, 25 are objected to as being dependent upon a rejected base claim. Applicants believe they have overcome the rejections to the base claims from which claims 6-9 and 25 depend, thereby obviating this objection. Reconsideration and withdrawal of the objection is respectfully requested.

Overall Summary and Conclusion

In view of the foregoing amendments and remarks, Applicants respectfully submit that claims 1-10, 14-18, and 20-29 set forth an invention that is new, useful, and unobvious, and which is therefore deserving of patent protection. Passage to issue of the present application is believed to be in order, and is respectfully requested.

Please charge any fees or credit any overpayment in connection with this application which may be required by this or any related paper to Deposit Account No. 05-0840.

If the Examiner has any questions, or would like to discuss any matters in connection with this application, he is invited to contact the undersigned at (317) 433-9829.

Respectfully submitted,

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Feb. 5, 2003

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Attachments:

1. Schaus 132 Declaration
2. 2 background reference articles
3. Streitwieser & Heathcock reference pages

NeuroReport 8, 2237–2240 (1997)

5-HT_{1F} receptor agonists inhibit neurogenic dural inflammation in guinea pigs

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THE serotonin (5-HT) receptor subtype mediating inhibition of neurogenic dural inflammation in guinea pigs was investigated using a series of serotonin agonists with differing affinities for the 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors. When agonist potencies for inhibiting neurogenic inflammation were compared with affinities for these receptor subtypes, a significant positive correlation was seen only with the 5-HT_{1F} receptor. The potency of agonists in inhibiting adenylate cyclase in cells transfected with human 5-HT_{1F} receptor was also highly correlated with their potency in the animal model of migraine. *In situ* hybridization demonstrated 5-HT_{1F} receptor mRNA in guinea pig trigeminal ganglion neurons. These data suggest that the 5-HT_{1F} receptor is a rational target for migraine therapeutics.

Key words: Migraine; Receptor localization; Saphenous vein; Serotonin receptor; Trigeminal; Vasoconstriction

Introduction

According to the neurogenic dural inflammation hypothesis of migraine pain,[1] an unknown stimulus activates the trigeminal nerve causing neurotransmitter release from its peripheral branches innervating the dura, a pain-sensitive intracranial membrane, initiating an inflammatory cascade which may sensitize dural nociceptors. Another consequence of trigeminal activation is the transmission of pain information to the central nervous system via the central branches of these same trigeminal neurons.[2] Thus, inhibition of trigeminal neurotransmitter release blocks both peripheral neurogenic inflammation and central pain transmission, mechanisms proposed to be modulated by 5-HT_{1B} receptors in the rat and by 5-HT_{1D} receptors in the guinea pig.[3,4]

Inhibition of dural nociceptive afferents in the periphery can be readily measured using the neurogenic dural inflammation model of migraine pain, in which trigeminal neurotransmitter release (substance P) is indirectly measured by quantifying plasma protein extravasation in the dura following electrical stimulation of the trigeminal ganglion in anesthetized guinea pigs.[5] To examine the role of 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors in the serotonergic inhibition of dural pain processes, the potency of several structurally diverse serotonin agonists with variable affinities for each of the targeted receptors was determined in this animal model. In addition to receptor binding affinities, the *in vitro* potency of these compounds to inhibit the formation of cyclic AMP in cells expressing the 5-HT_{1F} receptor was determined and compared to potency in the animal model of migraine pain.

Materials and Methods

Drugs: LY302148 (5-fluoro-3-[1-[2-(1-methyl-1*H*-pyrazol-4-yl)ethyl]-4-piper-idinyl]-1*H*-indole), LY306258 (3-dimethylamino-2,3,4,9-tetrahydro-1H-carbazol-6-ol), LY334370 (4-fluoro-*N*-[3-(1-methyl-4-piperidinyl)-1*H*-indol-5-yl]-benzamide), naratriptan, zolmitriptan and rizatriptan were synthesized at Eli Lilly and Co., Indianapolis, IN. Sumatriptan was purchased as a saline solution for injection from a local pharmacy. Dihydroergotamine (DHE), Evans Blue and pentobarbital were purchased from Sigma Chemical Company, St. Louis, MO. L-694,247 was purchased from Research Biochemicals International (RBI), Natick, MA.

Animal model of neurogenic dural inflammation: Animal experimentation using this model was conducted in accordance with the institutional guidelines of Eli Lilly & Co., an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited organization. The methods used have been reported in detail elsewhere.[6] Male,

Hartley guinea pigs were deeply anesthetized with sodium pentobarbital and placed in a stereotaxic frame. Two pairs of stimulating electrodes were placed to bracket both trigeminal ganglia. Test compounds were injected i.v., followed ~8 min later by Evans Blue (50 mg/kg, i.v.), a fluorescent dye that binds to plasma proteins and functions as a marker for protein extravasation. Exactly 10 min after injection of the test compound, the left trigeminal ganglion was stimulated for 3 min at a current intensity of 1.0 mA (5 Hz, 4 ms duration). Fifteen minutes following trigeminal stimulation, the animals were killed by exsanguination with 40 ml of saline. Dural membrane samples were removed from both hemispheres, rinsed with water, and spread flat on microscope slides. Once dried, the tissues were coverslipped with a 70% glycerol in water solution. A fluorescence microscope equipped with a grating monochromator and a spectrophotometer was used to quantify the amount of Evans Blue dye in each sample. An excitation wavelength of approximately 535 nm was utilized and the emission intensity at 600 nm was measured. The tissue fluorescence intensity was quantified as a measure of plasma protein extravasation. Intravenous dose–response curves in the neurogenic dural inflammation model were generated for each compound and the dose that produced a 50% inhibition of neurogenic inflammation (ID_{50}) was estimated from these curves.

Radioligand binding: Binding studies were conducted using membranes from LM(tk⁻) cell lines stably transfected with either the cloned human 5 HT_{1B}, 5-HT_{1D} or 5-HT_{1F} receptors, using tritiated 5-HT as the radioligand as described in Refs 7 and 8.

Cyclic AMP inhibition in cells: Inhibition of forskolin-stimulated cyclic AMP accumulation was measured in LM(tk⁻) cell lines stably transfected with the cloned human 5 HT_{1F} receptor as described in Ref. 9.

In situ *hybridization:* Male Hartley guinea pigs (301–350 g) were euthanized using CO_2, decapitated and their brains immediately removed and frozen by immersion in isopentane on dry ice. Sections were cut at 11 μm with a cryostat, thaw mounted onto poly-L-lysine coated slides then stored at −80°C until use. Antisense and sense oligonucleotides directed at the second extracellular loop of the guinea pig 5 HT_{1F} receptor were synthesized on a Cyclone Plus DNA Synthesizer (Milligen/Bioresearch). The sequence of the sense probe was 5′-CACCAGGGGACAAGCCGAGATGATGAGTGCATCATCAAGCATGAC-3′ and that of the antisense probe was 5′-GTCATGCTTGATGATGCACTCATCATCTCGGCTTGTCCCCTGGTG-3′.[8] The probes were labeled and the *in situ* hybridization was carried out as described previously.[10]

Contraction of rabbit saphenous vein: Male New Zealand White rabbits were sacrificed by a lethal dose of pentobarbital. The saphenous vein was dissected free of other tissues and placed in Krebs–bicarbonate buffer where ring preparations were processed. Tissues were mounted in organ baths containing 10 ml of Krebs solution maintained at 37°C and aerated with 95% O_2 and 5% CO_2. An initial optimum resting force of 4 g was applied and isometric contractions were recorded. Tissues were allowed to equilibrate for 1–2 h before cumulative agonist concentration-response curves were generated. Values used represent means of 3–13 individual experiments.

Results

Each of the compounds studied inhibited dural plasma protein extravasation after intravenous administration with a rank order of potency: LY334370 > naratriptan > LY302148 > LY306258 = zolmitriptan (BW-311c90) > dihydroergotamine > sumatriptan > rizatriptan (MK-462) >> L-694,247 (Table 1). Correlation of the i.v. ID_{50} values of these compounds in the neurogenic inflammation model with affinity at each of the serotonin receptors revealed a highly significant ($r = 0.94$) correlation with affinity for the 5-HT_{1F} receptor (Fig. 1a–c). No significant correlation existed between potency in the dural inflammation model and affinity for 5-HT_{1B} ($r = 0.38$) or 5-HT_{1D} ($r = 0.45$) receptors. When the *in vivo* potencies of these compounds in the inflammation model were compared with their *in vitro* potency to inhibit forskolin-induced cyclic AMP accumulation in cells transfected with the human 5-HT_{1F} receptor, a highly significant correlation was again apparent ($r = 0.93$, Fig. 1d).

In situ hybridization using radiolabled antisense probes for 5-HT_{1F} receptor message visualized with liquid emulsion autoradiography demonstrated silver grains over neurons of the guinea pig trigeminal ganglion (Fig. 2) Little hybridization signal was observed over ganglion cells hybridized using the sense oligonucleotide.

The EC_{50} for contracting rabbit saphenous vein tissue was determined for the compounds in Table 1. There was a strong positive correlation between the EC_{50} values for this series of compounds to produce rabbit saphenous vein contraction and their affinity at the 5-HT_{1B} ($r = 0.93$, $p < 0.003$) and the 5-HT_{1D} ($r = 0.93$, $p < 0.003$) receptors. For these same compounds, there was no significant positive correlation between 5-HT_{1F} affinity and potency in the saphenous vein assay.

Table 1. Receptor binding and potency data for selected serotonin agonists.

Compound	5-HT_{1B} binding (pK_i)	5-HT_{1D} binding (pK_i)	5-HT_{1F} binding (pK_i)	5-HT_{1F} (pEC_{50})	Extravasation potency (−log ID_{50})	Rabbit saphenous vein contraction (pEC_{50})	Rabbit saphenous vein contraction (% max)
LY334370	6.87	6.86	8.78	8.82	13.19	<4.00	1
LY302148	7.27	7.66	8.60	8.62	12.10	6.11	12
Naratriptan	8.48	8.55	8.35	8.66	12.42	6.31	22
LY306258	5.77	6.10	7.99	8.03	11.06	<4.00	12
Zolmitriptan	8.33	9.03	7.59	8.15	11.06	7.01	56
Sumitriptan	8.02	8.32	7.59	7.46	10.36	6.00	44
DHE	9.22	9.40	6.56	6.91	10.63	7.77	21
Rizatriptan	8.00	8.42	6.55	7.60	9.66	6.15	34
L-694,247	8.92	8.57	<5.30	<5.30	7.66	7.53	39

The pK_i values of selected compounds at the human 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors are listed in columns 2–4. The pEC_{50} values of these compounds to inhibit the forskolin-stimulated increases in cyclic AMP in cells transfected with the human 5-HT_{1F} receptor are shown in column 5. Column 6 contains ID_{50} values (expressed as −log M/kg) in the guinea pig neurogenic dural extravasation model of migraine. Column 7 lists pEC_{50} values for contracting the rabbit saphenous vein *in vitro*. Column 8 lists the maximal *in vitro* contraction caused by these compounds, defined as percent of the contraction produced by 67 mM KCl.

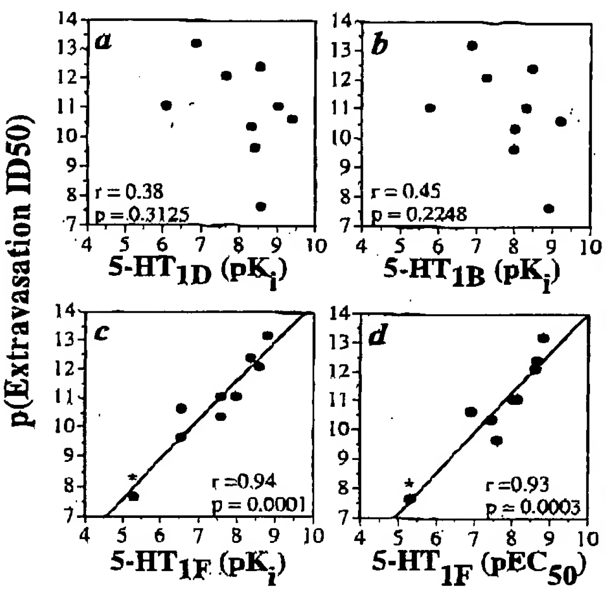

FIG. 1. Correlation analysis of binding and functional data with animal model results. (**a,b,c**) Correlation plots of the compounds in Table 1 comparing their receptor affinity (on the x-axis) and i.v. potency in the dural extravasation model (on the y-axis). (**d**) A similar analysis except that the x-axis represents the pEC_{50} values for the individual compounds in the whole cell cyclic AMP inhibition assay. The R value and P level are displayed for each correlation. The calculated line of best fit is displayed for statistically significant correlations. Asterisks indicate that the values plotted were the maximum concentrations tested. Without these points the r-values would be 0.88 and 0.84 and the *p*-levels would be 0.003 and 0.009 for (c) and (d), respectively.

FIG. 2. Localization of 5 HT_{1F} mRNA in guinea pig trigeminal ganglion. (**a**) High magnification brightfield photomicrograph of liquid emulsion autoradiography showing silver grains over neurons of the trigeminal ganglion hybridized with the antisense oligonucleotide. Labeled ganglion cells are indicated by the arrows. Bar = 50 μm. (**b**) Little hybridization signal was observed over ganglion cells hybridized using the sense oligonucleotide. Arrows indicate unlabeled ganglion cells.

Discussion

Sumatriptan is an efficacious agent approved for acute migraine treatment.[11,12] Although this serotonin receptor agonist was developed as a vasoconstrictor based on the vasodilatation theory of migraine,[13] sumatriptan also inhibits neurotransmitter release from trigeminal neurons involved in pain transmission.[14] It is unclear which of these actions relieve migraine, and which serotonin receptors are affected. While most of the consequences of sumatriptan administration have been ascribed to activation of

5-HT_{1B} or 5-HT_{1D} receptors,[15] it was also reported to activate the recently cloned 5-HT_{1F} receptor.[8] Taken together, our data argue that activation of the 5-HT_{1F} receptor, not the 5-HT_{1B} or 5-HT_{1D} receptors, is responsible for the efficacy of sumatriptan and second generation sumatriptan-like drugs in the guinea pig neurogenic dural inflammation model, an effect thought to be related to their anti-migraine activity. The 5-HT_{1B} and 5-HT_{1D} receptors appeared to play little role in this process since compounds with high affinity for these receptors, and low affinity for the 5-HT_{1F} receptor, demonstrated a potency proportional only to their 5-HT_{1F} receptor affinity. These data are in contrast to the apparent role of 5-HT_{1B} receptors in the mouse, as demonstrated by the inactivity of sumatriptan in transgenic animals lacking a 5-HT_{1B} receptor.[16] Using *in situ* hybridization, we established that guinea pig trigeminal neurons contain message for the 5-HT_{1F} receptor. The demonstration of significant levels of 5-HT_{1F} mRNA in the human trigeminal ganglion[17] is consistent with the possibility that sumatriptan itself acts through a 5-HT_{1F} receptor to exert its anti-migraine effects.

Because sumatriptan displayed high affinity for the 5-HT_{1B} and 5-HT_{1D} receptors[15] and was identified based on its ability to contract vascular tissue,[13] it was not surprising that sumatriptan also produced coronary arterial vasoconstriction.[18,19] In fact, several clinical reports have emerged documenting the cardiovascular liabilities of this agent.[19-21] Since compounds with high affinity and potency for the 5-HT_{1F} receptor did not contract this tissue, the $5\text{-HT}_{1\text{-like}}$ receptor that mediates vasoconstriction is clearly not the 5-HT_{1F} receptor. Furthermore, since selective, high affinity 5-HT_{1F} receptor agonists were exceptionally potent in the dural inflammation model of migraine but devoid of vasoconstrictor properties, selective 5-HT_{1F} receptor agonists may provide acute migraine therapies lacking vasoconstrictive liabilities.

Conclusion

Results in the neurogenic dural inflammation model of migraine demonstrate that selective 5-HT_{1F} receptor agonists inhibit the peripheral inflammatory processes proposed to occur during migraine pain. In addition, these data further suggest that 5-HT_{1F} receptor stimulation in the efferent central limb of this pathway may block the transmission of nociceptive information to the nucleus caudalis in the brainstem, which contains both mRNA for the 5-HT_{1F} receptor[22] and 5-HT_{1F} binding sites.[23] Inhibition of trigeminal-mediated peripheral inflammation and central pain transmission is the likely mechanism producing benefit in patients with migraine, an effect we now believe to be mediated by 5-HT_{1F} receptor activation. LY334370 is a selective, high affinity 5-HT_{1F} receptor agonist that potently inhibited dural inflammation but was devoid of the vasoconstrictor properties seen in current serotonin-based acute migraine therapies. Therefore, this compound provides a useful agent to test the hypothesis that serotonergic agonists relieve migraine pain via activation of 5-HT_{1F} receptors rather than vasoconstriction. This hypothesis is currently under investigation with the clinical evaluation of LY334370 in acute migraine pain.

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General Summary

Sumatriptan, a serotonin (5-HT) receptor agonist effective in treating acute migraine, inhibits neurotransmitter release from trigeminal neurons involved in pain transmission and also causes vasoconstriction. This vasoconstriction is the source of sometimes serious side effects. While most of the consequences of sumatriptan administration have been ascribed to activation of 5-HT_{1B} or 5-HT_{1D} receptors, it was also reported to activate the recently cloned 5-HT_{1F} receptor. To determine which receptor subtype mediates trigeminal inhibition, a series of serotonergic receptor agonists with differing selectivity toward these receptors was examined in the guinea pig neurogenic dural inflammation model of migraine. Unexpectedly, 5-HT_{1F} receptor affinity and activity and not 5-HT_{1B} or 5-HT_{1D} receptor affinity significantly correlated with potency in the migraine model. Selective, non-vasoconstrictive 5-HT_{1F} receptor agonists, as exemplified by LY334370, may represent a safer therapy for migraine pain.

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A fluorescence-based method for assessing dural protein extravasation induced by trigeminal ganglion stimulation

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Abstract

Neurogenic dural inflammation has been proposed as a source of pain during migraine. Unilateral electrical stimulation of the trigeminal ganglion causes the ipsilateral release of inflammatory neuropeptides and subsequent dural plasma protein extravasation, a component of neurogenic inflammation. We measured the amount of protein leaking into the dural tissue of guinea pigs following trigeminal ganglion stimulation by exploiting the complexation reaction of endogenous proteins with the fluorescent dye Evans Blue, instead of utilizing exogenous radiolabeled albumin as commonly done in the literature. The amount of Evans Blue trapped in dural tissue following electrical stimulation of the trigeminal ganglion was measured using a fluorescence microscope equipped with a spectrophotometer. This method utilized multiple measurements on each dura sample which resulted in very precise values using a small number of animals per point ($n = 3$). Sumatriptan and CP-122,288 were found to dose-dependently prevent neurogenic dural extravasation. The potencies of CP-122,288 and sumatriptan were found to be similar to those reported in the literature when similar experimental protocols were used. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Migraine; Fluorescence photomicroscopy; Electrical; Antidromic; Evans blue; Sumatriptan; CP-122,288

1. Introduction

Neurogenic dural inflammation has been proposed as a source of pain during migraine (Moskowitz et al., 1979; Hardebo, 1984; Moskowitz, 1984; Markowitz et al., 1987; Moskowitz, 1992). The dural membrane surrounding the brain is the source for the majority of intracranial pain afferents (Steiger et al., 1982; Mayberg et al., 1984). Dural inflammation is produced by the release of inflammatory neuropeptides (substance P, calcitonin gene related peptide, neurokinin A) from these sensory afferents in dural tissue (Lembeck and Holzer, 1979; Saria et al., 1985, 1986). A method for inducing dural inflammation was reported utilizing unilateral electrical stimulation of the trigeminal ganglion (Markowitz et al., 1987; Moskowitz, 1992). This stimulation caused antidromic depolarization and the unilateral release of inflammatory dural neuropeptides. These neuropeptides react with mast cells and blood vessel walls to cause ipsilateral dural protein extravasation from post-capillary venules (Moskowitz, 1992) and increased cerebral blood flow (Goadsby and Edvinsson, 1993).

Most groups investigating neurogenic dural inflammation have quantified protein extravasation using exogenous radiolabeled albumin (Saito et al., 1988; Buzzi et al., 1991; Huang et al., 1993; Moussaoui et al., 1993; O'Shaughnessy and Connor, 1993; Shepheard et al., 1993; Lee et al., 1994; Buzzi et al., 1995; Gupta et al., 1995). Values were expressed as the ratio of radioactivity in the dura from the stimulated versus the unstimulated side. Ergot alkaloids (Saito et al., 1988), sumatriptan (Huang et al., 1993; Lee and Moskowitz, 1993; Shepheard et al., 1995), acetylsalicylic acid (Buzzi et al., 1989) and several novel compounds (Shepheard

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et al., 1993; Lee et al., 1994; O'Shaughnessy and Connor, 1994; Gupta et al., 1995; Shepheard et al., 1995) have been shown to decrease or completely inhibit dural extravasati n following trigeminal ganglion stimulation, thus lowering the ratio to approximately one.

We modified this technique to allow quantification of endogenous protein leaking into the dural tissue by exploiting the complexation reaction of proteins with the fluorescent dye, Evans Blue (Rawson, 1943; Allen and Dralpvats, 1948). The dye was injected intravenously in guinea pigs prior to stimulation of the trigeminal ganglion, to label circulating proteins. Following unilateral ganglion stimulation, samples of dura overlying both hemispheres were removed, rinsed and mounted on a microscope slide. The amount of Evans Blue trapped in dural tissue was measured using a Zeiss fluorescence microscope equipped with a spectrophotometer. The ratio of the average amount of fluorescence in the dura from the stimulated side versus the unstimulated side was calculated. These modifications resulted in data being obtained faster with fewer animals and without the need for radioactive compounds. We have used this fluorescent technique to measure dural extravasation and evaluate the potency of sumatriptan and CP-122,288 to inhibit dural protein extravasation following intravenous administration.

2. Materials and methods

All compounds used in this study were dissolved in a 0.9% sodium chloride solution from Baxter Healthcare Corporation (Deerfield, IL). The Evans Blue dye and sodium pentobarbital were obtained from Sigma (St. Louis, MO). Sumatriptan (Imitrex™) was purchased from Cerenex Pharmaceuticals (Research Triangle Park, NC) as an injectable solution and diluted with saline to the desired concentrations. Glycerol was purchased from EM Science (Gibbstown, NJ) and diluted to a 70% solution with deionized water. CP-122,288 was prepared by standard synthetic chemistry methods.

Male Hartley guinea pigs (250–350 g, Charles River Laboratories, Wilmington, MA) were anesthetized with sodium pentobarbital (45 mg/kg, intraperitoneal) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) with the incisor bar set at −4.0 mm. Following a midline sagittal scalp incision, two pairs of bilateral holes were drilled through the skull (4 mm posteriorly and 3.2 and 5.2 mm laterally—all coordinates referenced to bregma). Pairs of stainless steel stimulating electrodes, insulated except at the tips (David Kopf Instruments, Tujunga, CA), were lowered through the holes in both hemispheres to a depth of 10.5 mm from dura.

2.1. Intravenous dosing studies

The femoral vein was exposed and a dose of the test compound was injected directly into the vein (1 ml/kg). Approximately 8 min later, a 50-mg/kg dose of Evans Blue was also injected intravenously. The Evans Blue complexed with circulating proteins and functioned as a marker for protein extravasation. Exactly 10 min after injection of the test compound, the left trigeminal ganglion was stimulated for 3 min at a current intensity of 1.0 mA (5 Hz, 4 msec duration) with a Model 273 potentiostat/galvanostat (EG&G Princeton Applied Research, Princeton, NJ).

2.2. Tissue collection

Immediately following trigeminal ganglion stimulation, the anesthetized animals were killed by perfusion exsanguination performed by injecting saline (40 ml, 1 ml/s) into the left ventricle of the heart after the right atrium had been cut. The descending aorta and the vena cava were clamped at the level of the diaphragm to enhance rostral perfusion.

The top of the skull was removed to allow the collection of the supratentorial dural membranes. The membrane samples were collected from both hemispheres, rinsed with deionized/distilled water, and spread flat on microscopic slides. Following drying at 37°C for approximately 15 min, the tissues were coverslipped with a 70% glycerol/water solution.

2.3. Quantification of protein extravasation

A fluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with a grating monochromator and a spectrophotometer was used to quantify the amount of Evans Blue dye in each sample. An excitation wavelength of 535 nm (band pass filter) was utilized and the emission intensity at 600 nm was determined. The microscope was equipped with a motorized stage and also interfaced with a personal computer. This facilitated the computer-controlled movement of the stage and fluorescence intensity was measured at 25 points (5 × 5 array of 500 μm steps) on each dural sample. The tissue immediately surrounding the electrode penetration sites was avoided for all measurements. The mean of the individual measurements on each dura sample was determined by the computer. All stage movements, fluorescent measurements, and calculations were performed by the software program Photan (Carl Zeiss, Thornwood, NY).

2.4. Data analysis

The extravasation induced by electrical stimulation of the trigeminal ganglion was an ipsilateral effect (i.e.

increased extravasation occurs on the side of the dura in which the trigeminal ganglion was stimulated). This allowed dura from the unstimulated hemisphere to be used as a control. The ratio of the average amount of extravasation (fluorescence) in the dura from the stimulated side compared to the unstimulated side dura was calculated for each animal. The individual ratios for three animals were averaged for each treatment. Saline controls yielded a ratio of approximately 1.8. In contrast, a compound at a dose which effectively prevented the extravasation in the dura from the stimulated side would generate a ratio of approximately 1.0. A dose-response curve was generated and the dose that inhibited the extravasation by 50% (ID_{50}) was approximated. The statistical significance of the differences between the ratios was assessed by ANOVA and Dunnett's test.

3. Results

Following unilateral electrical stimulation of the trigeminal ganglion, fluorescence analysis performed directly on the harvested dural membranes revealed a profound difference in the amount of the Evans Blue dye in the stimulated side compared to the unstimulated side of dura from saline-treated animals as shown in the photographs in Fig. 1A and B. The Evans Blue present in the tissues was measured by fluorescence microspectroscopy and the ratio of the fluorescence from the stimulated compared to unstimulated side was expressed as the extravasation ratio. For animals treated with intravenous saline, this ratio was 1.86 ± 0.07 (mean $\pm$ S.E.M., $n = 3$).

Intravenous pretreatment with sumatriptan produced significant dose-related decreases in the extravasation ratio at doses of 1.2 μg/kg ($p < 0.05$) and 12 μg/kg ($p < 0.01$) compared to saline controls (Fig. 2). An ID_{50} value (dose of compound which produced half maximal inhibition of extravasation) of 0.7 μg/kg was approximated from the dose-response curve. Fig. 2 also illustrates the dose-response curves for CP-122,288 given intravenously to guinea pigs. CP-122,288 decreased the extravasation ratio significantly at doses of 1 ng/kg ($p < 0.05$) and 10 ng/kg ($p < 0.001$) with an approximate ID_{50} value of 0.8 ng/kg (Fig. 2).

4. Discussion

We have modified a standard method used for quantifying plasma protein extravasation during neurogenic inflammation induced by electrical stimulation of the guinea pig trigeminal ganglion. We found that by using Evan Blue dye to label extravasated plasma proteins, fluorescence analysis could be performed directly on the harvested dural membranes instead of utilizing radiolabeled compounds or extracting the dye from the tissue prior to quantification. This new method revealed a profound difference in the amount of the Evans Blue dye in the stimulated side compared to the unstimulated side of dura from saline-treated animals as shown in the photographs in Fig. 1A and B. The amount of fluorescence in the tissue was proportional to the amount of protein that had leaked out of the blood vessels into the surrounding tissue. The Evans Blue present in the tissues was measured by fluorescence microspectroscopy and the ratio of the stimulated to unstimulated side was expressed as the extravasation ratio. For animals treated with intravenous saline, this ratio was 1.86 ± 0.07 (mean $\pm$ S.E.M., $n = 3$). The value obtained using saline as a control is similar to data reported previously using other methods in both guinea pigs and rats (Buzzi and Moskowitz, 1990; Shepheard et al., 1993; Lee et al., 1994). This confirms that the Evans Blue complexed with the endogenous protein passes into the tissue in a fashion similar to the exogenous radiolabeled albumin that previously had been utilized for this assay. In fact, when Markowitz, Saito and Moskowitz first published their technique using ^{125}I-BSA they concluded that their "isotopic technique reflects plasma extravasation in much the same way as Evans blue, and it validates comparison with previous work based on the Evans blue [extraction] technique" (Markowitz et al., 1987).

To validate this new technique, we determined the potency of the serotonin agonists sumatriptan and CP-122,288 to inhibit dural protein extravasation. Sumatriptan is efficacious in the clinical treatment of acute migraine pain and CP-122,288 is an experimental therapy.

Pretreatment of guinea pigs with intravenous sumatriptan produced significant dose-related decreases in the extravasation ratio with an approximate ID_{50} of 0.7 μg/kg (Fig. 2), which agreed very well with a published ID_{50} value for intravenous sumatriptan in guinea pigs of 1.2 μg/kg (Lee and Moskowitz, 1993).

Pretreatment with intravenous CP-122,288 potently inhibited protein extravasation with an ID_{50} value of 0.8 ng/kg which is also similar to the value of 0.5 ng/kg published by Lee and Moskowitz (1993) for CP-122,288.

The ID_{50} values approximated for sumatriptan and CP-122,288 using our fluorescence method and collecting dura immediately after stimulation compared favorably to those published by Lee and Moskowitz (1993) using their radiolabeled albumin method. This reproduction of published data, albeit with a small number of compounds, confirms the validity of this technique for the collection of protein extravasation data in the guinea pig dura. Furthermore, the similarity of the ID_{50} values also show that subtle differences in the stimulating electrode configuration and slight differences in stimulus duration do not have a significant effect on the data collected.

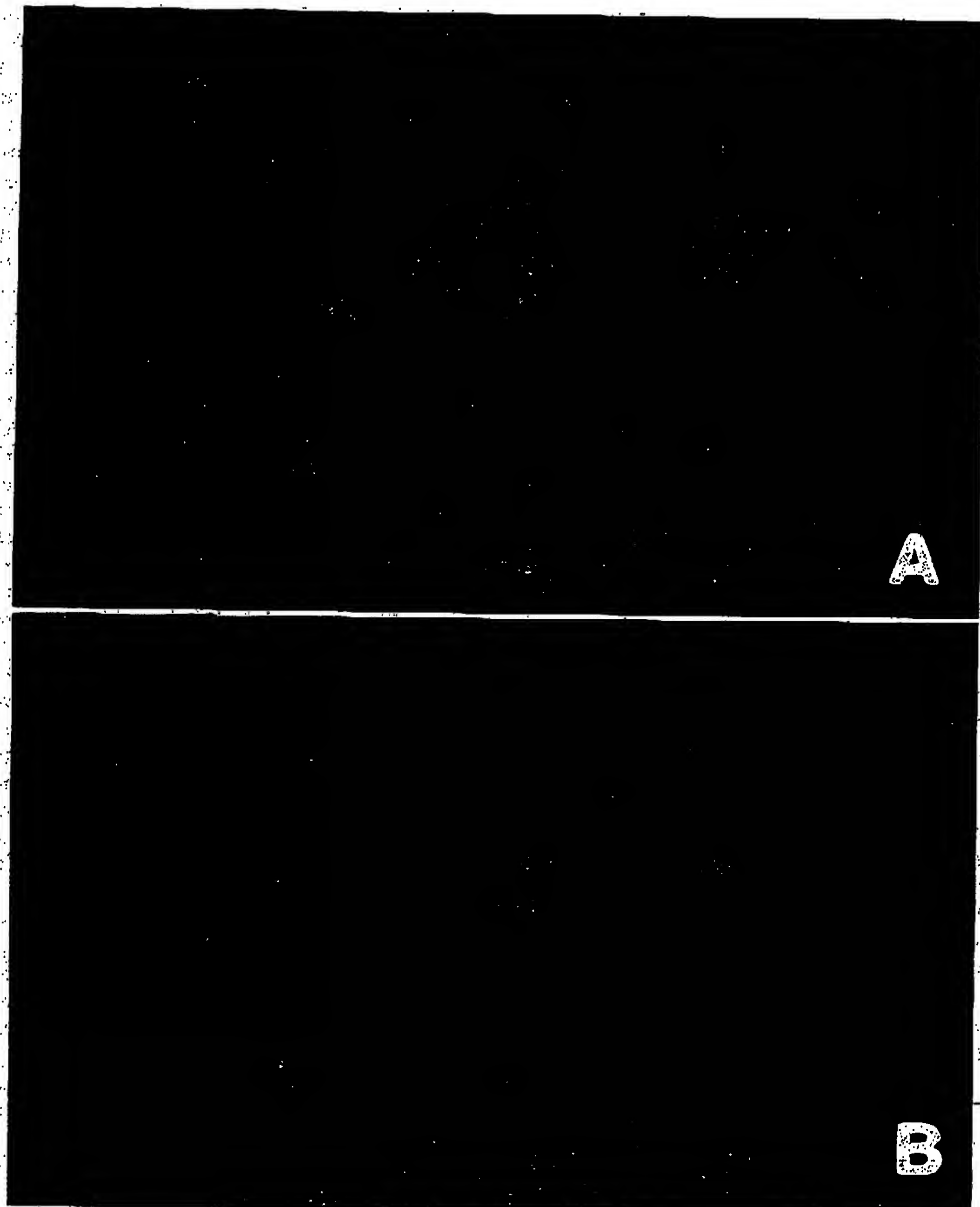

Fig. 1. Photomicrographs showing the fluorescence emission from Evans Blue in the dural tissue of a guinea pig. Both images were exposed for 0.82 s so the light image would be proportional to the amount of Evans Blue and plasma extravasation in the tissue. (A) Photomicrograph of Evans Blue fluorescence in the dura from the control (unstimulated) side. (B) Photomicrograph of Evans Blue fluorescence in the dura from the stimulated side.

This assay has several advantages over traditional methods used in the literature. Importantly, this assay does not require the use of ^{125}I-labeled bovine serum albumin which is more expensive than Evans Blue when considering purchase and disposal. Data can also be collected more quickly with this assay method because the quantification of extravasation is performed directly on the tissue without extraction. Data can routinely be analyzed within 15 min of tissue collection. In contrast, an overnight extraction or drying of tissue is required prior to quantifying the marker compounds, whether fluorescent or radioactive, with other methods. The method outlined in this paper also required fewer animals per data point to show significant drug effects.

Our method routinely required three animals per point, compared to 5–16 (Buzzi et al., 1991; Huang et al., 1993; Lee and Moskowitz, 1993; Shepheard et al., 1993, 1995) required of other methods. This decrease in the number of animals required to show significance results in substantial cost savings, and more importantly uses less animals per experiment which is a high priority within our institution and others. The multiple fluorescence emission measurements ($n = 25$) performed directly on each tissue sample, coupled with averaging, most likely is the source of this advantage. Literature techniques perform a single fluorescence, absorbance or radioactivity measurement on a tissue extract, not the actual tissue itself. This measurement is then divided by the weight of the tissue sample (wet or dry) to normalize the data. Weighing of the tissue is also a potential source of error due to varying degrees of tissue dryness at the time of weighing. Also, our use of stimulating electrode pairs (separated by 2 mm) implanted in a manner to bracket the trigeminal ganglion instead of a single electrode with concentric conductors implanted directly into the trigeminal ganglion also seemed to contribute to the reproducibility of stimulation effects (i.e. extravasation), but not the ultimate precision of the calculated ID_{50} values as mentioned previously.

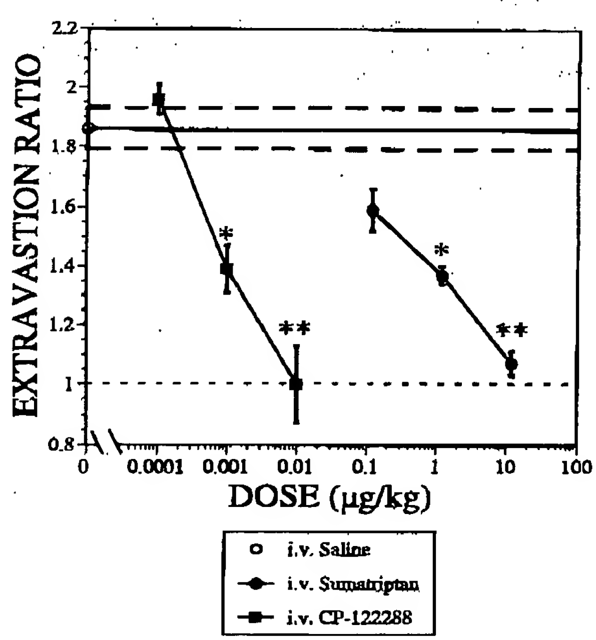

Fig. 2. Stimulation-induced plasma extravasation in guinea pigs following intravenous treatment with sumatriptan and CP-122,288. The extravasation ratio is the average fluorescence intensity of the dura from the stimulated side divided by the average fluorescence intensity of the dura from the unstimulated side. A ratio of one would indicate a completely effective treatment. Results reported as the mean ± S.E.M. ($n = 3$). Statistical analysis was performed by comparison to a saline control using ANOVA and Dunnett's test; (*$p < 0.05$, **$p < 0.001$).

One potential disadvantage to this technique is the requirement of the fluorescence microscope with spectrophotometric capabilities. In limited trials (data not shown), similar extravasation ratios have been obtained by utilizing a standard fluorescence microscope having a camera with automatic exposure control. With this equipment the extravasation ratio is derived by calculating the ratio of the exposure time for photographing the unstimulated side of the dura divided by the exposure time for photographing the stimulated side. This ratio (unstimulated/stimulated) is the inverse of the ratio calculated when measuring the fluorescence directly because the intensity of fluorescence emission and exposure time calculated by the camera should be reciprocal.

5. Conclusion

In conclusion, a method has been outlined which uses fluorescence to measure dural protein extravasation in guinea pigs following ipsilateral electrical stimulation of the trigeminal ganglion. Dural protein extravasation is one component of neurogenic inflammation proposed to be associated with migraine pain. This method has advantages over methods currently in the literature. Substantial cost savings can be realized by using cheaper reagents which are safer and easier to dispose of, by using fewer animals, and by generating data more quickly. Sumatriptan and CP-122,288 were effective in this model, with approximate ID_{50} values similar to data obtained by other methods in the literature.

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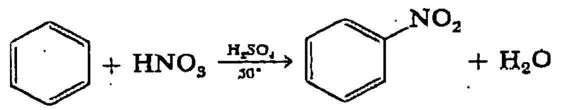
system, is much faster than its reaction with water. With ordinary carbonium ions, reaction with a nucleophilic species is an important reaction, but with such carbonium ions, elimination of a proton does not have the extraordinary driving force of the formation of an aromatic ring.

C. *Nitration*

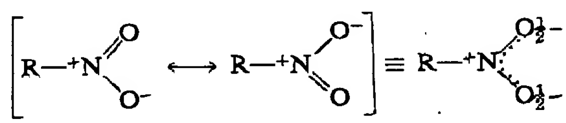
The reaction of alkenes with nitric acid is not a generally useful reaction. Addition of nitric acid to the double bond is accompanied by more or less oxidation. However, benzene is quite stable to most oxidizing agents, and its reaction with nitric acid is an important organic reaction. Actually, the nitrating reagent generally used is a mixture of concentrated nitric acid and sulfuric acid.



nitrobenzene

To a flask containing 65 g of benzene is added a mixture of 110 ml of conc. H_2SO_4 and 85 ml of conc. HNO_3. The acid mixture is added in portions so that the temperature does not exceed 50°. After all of the acid has been added, the reaction mixture is cooled and the oily nitrobenzene layer is separated, washed, and distilled. The yield of pure product is 85–88 g (83–86%).

The nitro group is an important functional group in aromatic chemistry because it may be converted into many other functional groups. The nitration reaction thus provides a route to many substituted aromatic compounds. The chemistry of the nitro group will be detailed in later chapters (Sections 27.8.C, 32.1). Many properties of the nitro group can be interpreted on the basis of a resonance hybrid of two Lewis structures:



In these structures, the O—N—O system is seen to have an allylic anion type of π system.

In a mixture of nitric and sulfuric acids, an equilibrium is established in which many species are present. One of these species is the nitronium ion, NO_2^+, which has been detected by spectroscopic methods. In the mixture of acids, it is produced by a process in which sulfuric acid functions as an acid and nitric acid functions as a base.

$$\text{(1)}\qquad H_2SO_4 + HONO_2 \rightleftharpoons H_2\overset{+}{O}NO_2 + HSO_4^-$$

$$\text{(2)}\qquad H_2\overset{+}{O}NO_2 + H_2SO_4 \rightleftharpoons H_3O^+ + {}^+NO_2 + HSO_4^-$$

$$2\,H_2SO_4 + HONO_2 \rightleftharpoons H_3O^+ + NO_2^+ + 2\,HSO_4^-$$

The structure of nitronium ion is known from spectroscopic measurements. It is related to the isoelectronic compound, carbon dioxide. The molecule is linear, and is a powerful electrophilic reagent.

$$O{=}\overset{+}{N}{=}O$$

nitronium ion

rdinary carbonium ions,
eaction, but with such
ie extraordinary driving

nerally useful reaction.
anied by more or less
idizing agents, and its
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: acid and sulfuric acid.

· H_2O

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$O^{\frac{1}{2}-}$

$O^{\frac{1}{2}-}$

an allylic anion type

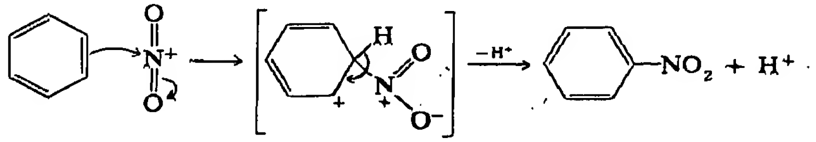
is established in which
nium ion, NO_2^+, which
of acids, it is produced
d nitric acid functions

HSO_4^-

2 HSO_4^-

opic measurements. It
he molecule is linear,

It reacts directly with benzene to give a pentadienyl cation intermediate.

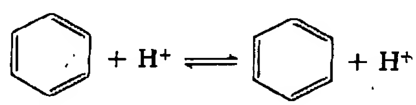


Note that reaction occurs on nitrogen rather than oxygen.

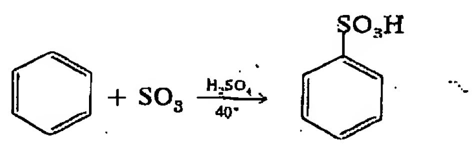
Reaction at oxygen gives a nitrite compound, R—O—NO. Nitrites are unstable under such strongly acidic conditions and decompose to products containing C—O bonds. These oxidation products react further to give highly colored polymeric compounds. The formation of more or less tarry byproducts is a usual side reaction in most aromatic nitration reactions.

D. *Sulfonation*

In the reactions of alkenes with sulfuric acid, the acid acts primarily as a protonating reagent to produce a carbonium ion, which reacts with nucleophiles present (Sections 12.6.B under Addition of HX and 12.6.F). We have seen that benzene itself undergoes protonation in strong sulfuric acid (Section 21.2.B). However, unless such a reaction is followed by use of a hydrogen isotope, it remains an *invisible* reaction.



With strong sulfuric acid, double bonds can react with the sulfur trioxide present. Such reactions of alkenes frequently result in oxidation of the organic material with concommitant reduction of sulfur trioxide to sulfur dioxide. Such reactions are not usually useful reactions of alkenes, although some exceptional cases do exist. The reaction of benzene with sulfur trioxide is a useful and important reaction. Sulfur trioxide is an electrophilic reagent and it reacts with benzene to give benzenesulfonic acid, the product of a sulfonation reaction. The reaction is usually carried out with a solution of sulfur trioxide in sulfuric acid, known as fuming sulfuric acid.



benzenesulfonic acid

Sulfur trioxide, SO_3, exists in several allotropic forms. The so-called α and β forms are polymers that form long fibrous needles. The γ form is a liquid monomer available commercially with an inhibitor to prevent polymerization. Sulfur trioxide is prepared by the catalytic oxidation of sulfur dioxide with oxygen. Sulfur trioxide is the anhydride of sulfuric acid and reacts vigorously with water with evolution of much heat. The reaction with heavy water, D_2O, is used to prepare D_2SO_4. Sulfuric acid is prepared commercially by dissolving sulfur trioxide in water to produce "fuming sulfuric acid." Commercial fuming sulfuric acid contains 7–8% of SO_3. Dilution with water gives ordinary concentrated sulfuric acid.